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Decreased Diversity of Hepatitis C Virus Quasispecies During Bone Marrow Transplantation

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To elucidate the role of host immune status in the evolution and complexity of hepatitis C virus (HCV) quasispecies, three chronic HCV-infected patients who underwent bone marrow transplantation (BMT) were studied. The three transplanted patients' sera were sampled at pre-BMT, 3 months after BMT, and 12 months after BMT and the nucleotide diversity and substitution of the hypervariable region (HVR) of HCV quasispecies were analyzed. The nucleotide diversity was high at the pre-BMT period (28.2–43.4 × 10^{-2} nucleotide difference/site). HVR of HCV quasispecies then became homogeneous in the first 3 months after BMT (0.11–6.40 × 10^{-2} nucleotide difference/site). The nucleotide diversity of HVR at 12 months after BMT of all three patients was higher than that of 3 months after BMT but still lower than that of pre-BMT (2.09–6.40 × 10^{-2} nucleotide difference/site). The analysis on nucleotide substitution rate showed a higher value between pre-BMT and 3 months after BMT (0.624–0.708 nucleotide difference/site per year) than that between 3 months and 12 months after BMT (0.072–0.127 nucleotide difference/site per year). HCV RNA titer decreased when the host had a low white cell count and increased accordingly. It was concluded that the evolution of HVR of HCV quasispecies related to the immune status of the host during BMT: after immunosuppression, an initial increase of viral populations was followed by the emergence of a dominant strain while the quasispecies gradually recovered as the immunity of the host gained its competence. ***J. Med. Virol. 58:132–138, 1999.***

KEY WORDS: hepatitis C virus; quasispecies; bone marrow transplantation

INTRODUCTION

Hepatitis C virus (HCV), like most RNA virus, replicates by an error-prone RNA polymerase lacking proofreading activity and circulates as a heterogeneous mixture of closely related but different genomes. HCV usually consists of a major sequence and a large spectrum of minor variants and this is referred to as a quasispecies nature [Martell et al., 1992]. This permits HCV to escape host immune surveillance easily [Shimizu et al., 1994] and makes the development of a vaccine difficult. The degree of nucleotide diversity of HCV genome, especially in the hypervariable region (HVR), which is one of the target of host immune selection [Weiner et al., 1992; Choo et al., 1994; Zibert et al., 1995], varies with the progression of chronic liver disease [Kurosaki et al., 1993; Honda et al., 1994]. The complexity of quasispecies has been reported to be related to the effectiveness of interferon therapy [Kanazawa et al., 1994].

Since its identification in 1989, HCV has been recognized as the main causative agent of posttransfusion non-A, non-B hepatitis [Choo et al., 1989]. Patients with hematological diseases are highly susceptible to HCV infection because they undergo transfusion frequently. Those patients also undergo bone marrow transplantation (BMT) frequently to cure their underlying diseases.

Patients undergoing organ transplantation(s) are immunocompromised and serve as good candidates to observe the evolution of HCV genome in individuals with low or minimal host immune pressure. It has been

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shown that after liver transplantation, the complexity of HCV quasispecies decreases [Martell et al., 1994; Gretch et al., 1996; Lawal et al, 1997], but that observation may simply reflect the short interval between the time of graft infection and sequence analysis. Little information about the changes of complexity of HCV quasispecies in patients undergoing transplantation other than the liver is available. Thus, studies in HCV-infected patients with organ transplantation other than liver may further elucidate the nature of HCV quasispecies evolution, because the heavily viral-loaded liver has not been removed and HCV may flourish under immunosuppression. The study of HCV quasispecies during this period may allow us to study the influence of host immune status on the evolution of viral quasispecies and provide some clues for viral pathogenesis.

PATIENTS AND METHODS

Three HCV-infected patients who underwent BMT and one HCV-infected patient without BMT were enrolled. The last patient served as a reference. Their sera for analysis were sampled just prior to BMT, 3 months, and 1 year after BMT. BMT protocol was described previously [Lin and Lin, 1989]. The conditioning chemotherapy, usually including busulfan and cyclophosphamide, was an immunosuppressive regime administered to the host 1 week before BMT. The host received the donor's bone marrow cells at day 0.

Detection of HCV RNA and Molecular Cloning of HVR of HCV cDNA

Serum HCV RNA was extracted by the single-step method [Chomczynski and Sacchi, 1987] and then processed into the reverse transcription-polymerase chain reaction (RT-PCR) procedure as described previously [Ni et al., 1994]. The outer primers are: 5′-ATGGCTTGGGACATGATGATGAACTGGT-3′ (nucleotide position 952–979), 5′-GTAGTGCCAG-CAATAAGGCC-3′ (nucleotide position 1467–1448); the inner primers are: 5′-TTAGTCGACTGGGGAGTCTG-GCGGGC-3′ (nucleotide position 1055–1074), and 5′-TTGCATGCCAGCTGCCATTGGTGTT-3′ (nucleotide position 1263–1244). The underlined sequences are SalI and SphI sites, respectively. The expected size of nested PCR product covering the HVR was 209 bp. The PCR product was then purified by phenol/chloroform treatment and precipitated by ethanol. The purified cDNA fragment was then subjected to SalI and SphI restriction enzyme digestion and ligated with pGEM-3Z (Promega, Madison, WI) with T_4 DNA ligase (Boehringer Mannheim, Germany) at 16°C for more than 4 hr. The recombinant plasmid DNA was transformed into competent cells, JM109 (Promega). The colonies were kept in a master plate for sequencing use.

Sequencing

Eight clones from the HCV HVR cDNA library of each serum sample were picked randomly and sequenced by the Amplicycle sequence kit (Perkin-Elmer, Norwalk, CT) based on the Sanger method. The sequence primer was the antisense inner primer referred to above. [α-^{33}P]dATP was incorporated in the cycle sequence reaction by Taq polymerase. The sequencing reaction samples then run on a 6% polyacrylamide gel. After electrophoresis, the gel was dried and autoradiographed at room temperature for more than 24 hr.

Sequence Analysis

The nucleotide diversity (π) in each set of sequences, that is, eight clones from each serum sample, was calculated as the average number of nucleotide differences per site of all possible comparisons of two sequences. It is defined as

$$\pi_x = \Sigma_{ij} x_i x_j \pi_{ij}$$

in which x_i is the frequency of the ith sequence in the population and π_{ij} is the number of nucleotide differences per nucleotide site between the ith and jth sequences [Nei and Li, 1979]. The amino acid diversity was calculated according to the same formula, however, in which π_{ij} is the number of amino acid differences per amino acid site between the ith and jth sequences.

The net nucleotide substitutions (δ) between two populations of sequences was estimated using the mathematical model as described by Nei and Li [1979]:

$$\delta = \pi_{xy} - (\pi_x + \pi_y)/2$$

in which $\pi_{xy} = \Sigma_{ij} x_i y_j \pi_{ij}$. The nucleotide substitution rate is δ/year. The amino acid sequence and the number of different amino acid sequences were also recorded.

Quantitation of HCV RNA by Competitive PCR

The method was described in a previous report [Lin et al., 1994]. Briefly, we first constructed a plasmid by inserting PCR fragments of the 5′-noncoding region (nucleotide position −341 to −1 of HCV) and the core region (nucleotide position 1 to 356 of HCV) into EcoRI and HindIII sites of pGEM-4 (Promega), respectively, by blunt end ligation. After in vitro transcription, the final competitor, consisting of the 5′-noncoding region, followed by an internal 56-bp fragment derived from the pGEM-4 polylinker and the core sequence, was obtained. Various concentrations of competitor RNAs were mixed with the isolated serum RNA to run nested PCR. The sequence of primers and the PCR condition were described previously [Lin et al., 1994]. The expected sizes of the nested PCR products for the wild type HCV and for the competitor RNA were 331 and 387 bp, respectively. After electrophoresis into a 2.5% agarose gel and by ethidium bromide staining, the

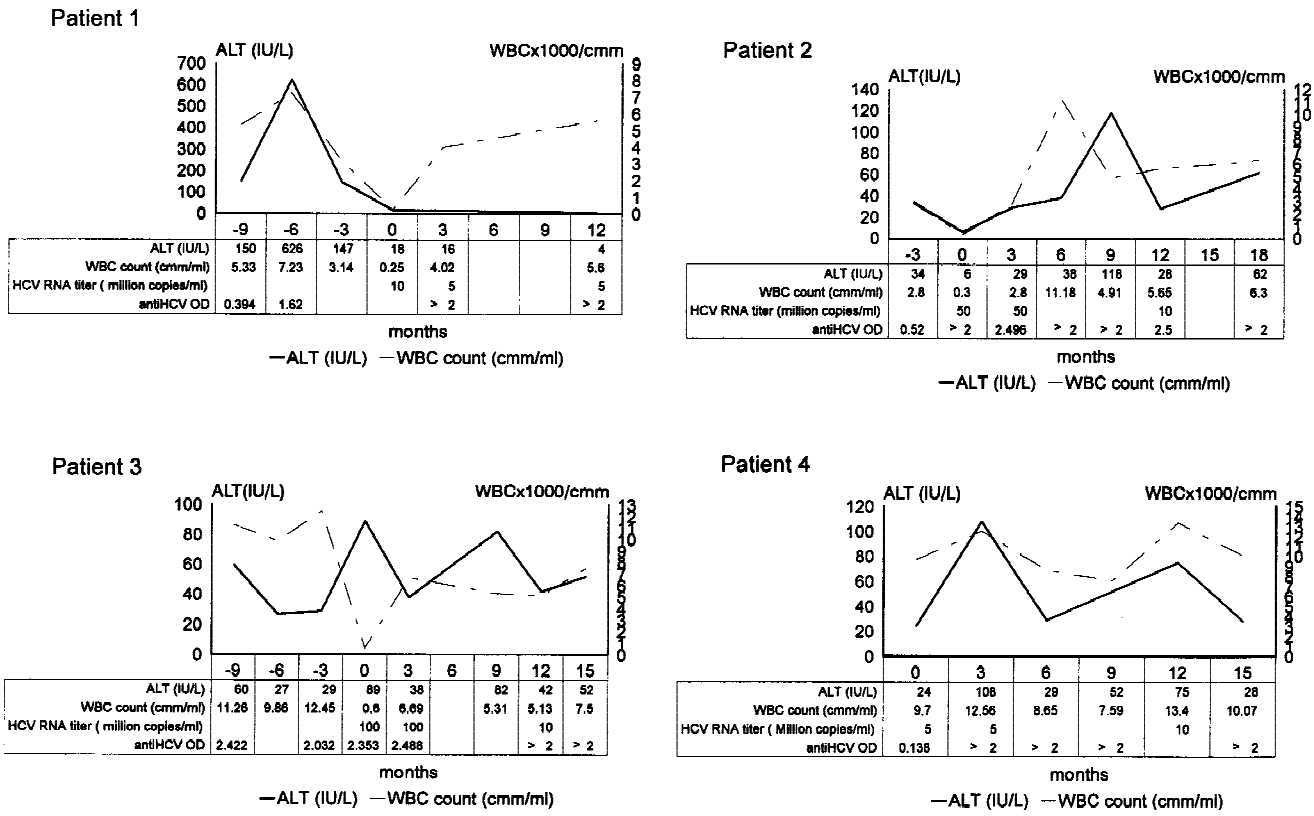

Fig. 1. The time course of alanine transaminase (ALT; solid line) and white blood cell count (dash line) changes of the four patients. Serum samples were withdrawn for sequence analysis at 0 (before bone marrow transplantation), 3, and 12 months after bone marrow transplantation. Hepatitis C virus (HCV) RNA titer and the optical density (OD) value of anti-HCV enzyme immunoassay (EIA) method were also recorded.

amount of wild type cDNA was determined to be equivalent to the concentration of competitor cDNA. All tests were done in duplicate.

RESULTS

Patient 1 (a 12-year-old girl) was a case of acute myelogenous leukemia. She was noted to have an episode of acute hepatitis after the induction phase of chemotherapy. She had received blood transfusion before chemotherapy. BMT was performed 1 year later when the leukemia was in remission stage. Busulfan (4.5 mg/kg per day for 2 days) and cyclophosphamide (50 mg/kg per day for 2 days) were administered 1 week before BMT as the conditioning therapy. The donor was her father. She died of recurrent leukemia 1 year later.

Patient 2 was a 13-year-old boy and was diagnosed with aplastic anemia. The patient had several episodes of severe bleeding and was transfused repeatedly after the onset of the disease. A successful BMT was carried out 3 months after the diagnosis. Pre-BMT conditioning therapy included cyclophosphamide (50 mg/kg per day for 2 days), and antithymocyte globulin (30 mg/kg for 3 days). His sister was an human leukocyte antigen (HLA)-compatible donor. An alanine transaminase (ALT) elevation was noted 1 month before BMT.

Patient 3 was a 12-year-old girl with β-thalassemia. She had received regular blood transfusions since the age of 11 months. Splenectomy was undertaken at the age of 6 years due to hypersplenism. The donor was her younger brother. Preconditioning therapy for BMT was busulfan (4.5 mg/kg per day for 2 days), cyclophosphamide (50 mg/kg per day for 2 days), and antithymocyte globulin (30 mg/kg for 3 days). An episode of jaundice and ALT flare-up was observed during BMT and another episode 6 months later.

Patient 4 was a 14-year-old thalassemic girl and did not receive BMT. However, she contracted HCV infection in the long-run regular blood transfusion course. Her nucleotide and amino acid diversity of HCV quasispecies were also calculated and provided a reference guide for comparison. The corresponding ALT and white blood cell (WBC) count changes are illustrated in Figure 1.

Before BMT, WBC counts were below 1,000/mm^3 in all transplanted patients because of the conditioning chemotherapy. WBC counts recovered normally at 3 and 12 months after BMT. The HVR nucleotide sequences as well as the amino acid sequences of the four patients were compared with Taiwan strain [Chen et al., 1992] (data not shown), whereas only Patient 1's nucleotide and amino acid sequences were listed as an illustrative case (Fig. 2). Most of the nucleotide substitution and amino acid sequence changes are concen-

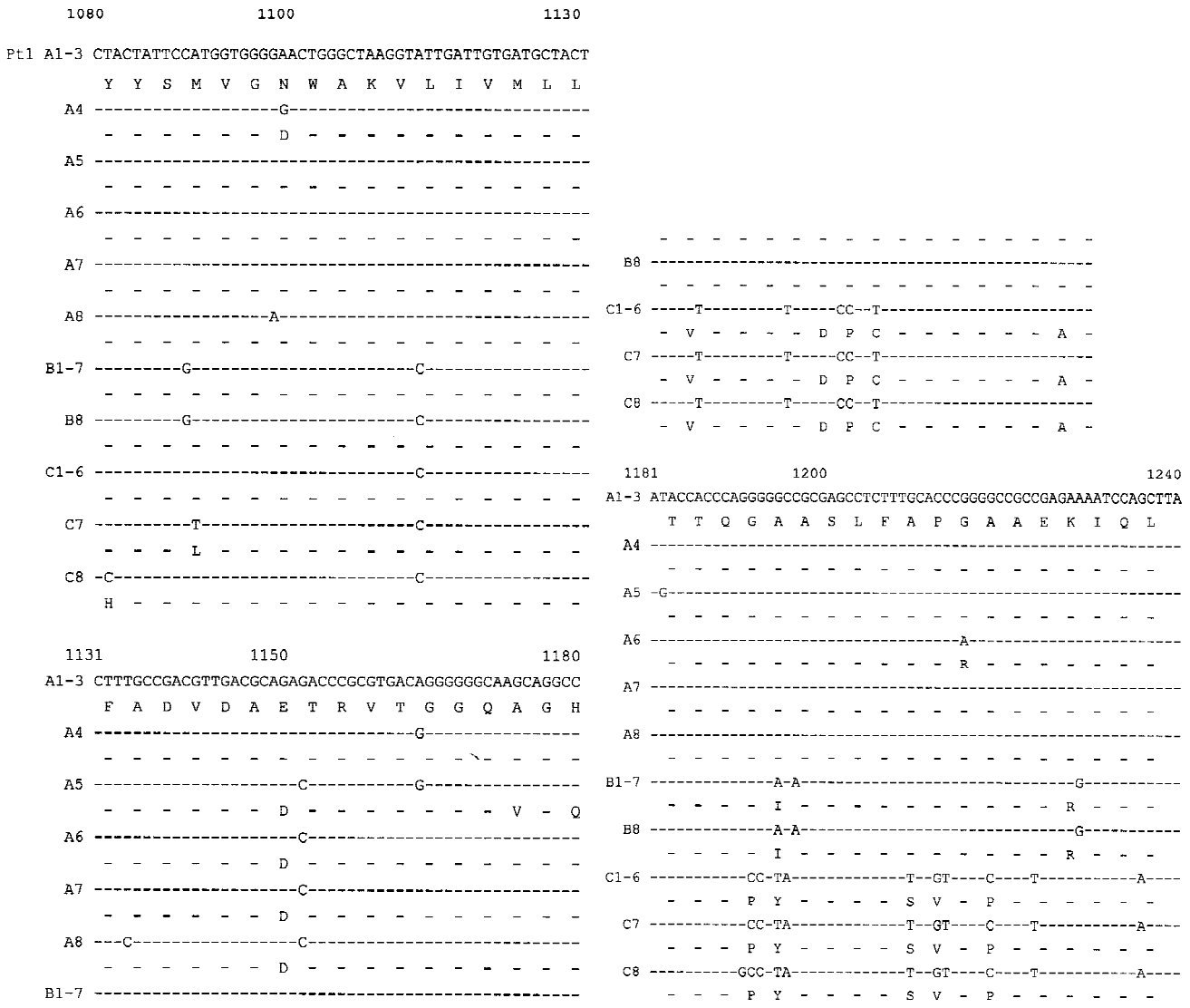

Fig. 2. The nucleotide and amino acid sequences of hypervariable region of hepatitis C virus cloned from the sera of patient 1. The amino acid was labelled at the first base of each codon. The nucleotide sequences were listed from position 1080 to 1240. The nomenclatures of the clones from each time point are **(A)** the sample taken before bone marrow transplantation, **(B)** the sample taken 3 months after bone marrow transplantation, and **(C)** denoted the sample taken 12 months after bone marrow transplantation. Each point had 8 randomly selected clones (Numbers 1–8).

trated in nucleotide position 1150–1220. The nucleotide diversity (π) of HVR of HCV for each serum sample is shown in Table I. The highest π was always in the pre-BMT time period when the host's immunity did not decline yet, whereas the lowest π tended to be in the 3 months after BMT when the immune response reached its nadir. The nucleotide substitutions (δ) and its rate of HVR between two populations of HCV clones from pre-BMT to 3 months after BMT, and between the two populations of HCV clones from 3 months to 1 year after BMT are described in Table II. The nucleotide sequence changes were markedly different from pre-BMT stage to 3 months after BMT. However, the changes lessened and the substitution rate was similar to that of the control when the patients' immune status recovered. Patient 4, who did not receive BMT, her nucleotide diversity, amino acid diversity (Table I) and substitution rate (Table II) were similar to the other patients' data 1 year after BMT.

HCV RNA titer and the optical density (OD) value of anti-HCV by enzyme immunoassay (EIA) was determined at those time points corresponding to each serum sample checked for sequence variability (Fig. 1). The amount of virus was the highest in the pre-BMT sample, during which time the patients received conditional therapy and the white cell count reached its nadir. The titer variation was within 10-fold during the 1-year follow-up period when the white cell count gradually recovered. Patient 4, who did not receive BMT, also showed a mild change over the 1-year period.

TABLE I. Sequence Complexity of HVR of HCV cDNA Quasispecies Before and 3 Months and 12 Months after BMT

Sample[a]	% of identical nucleic acid	No. of different nucleic acid sequences among 8 clones	π: nucleotide diversity ($\times 10^{-2}$/site)	% of identical amino acid	No. of different amino acid sequences among 8 clones	Amino acid diversity ($\times 10^{-2}$/site)
Pt 1-0M	96–99	6	43.48	94–99	4	2.50
Pt 1-3M	99	2	0.11	100	1	0
Pt 1-12M	98–99	3	2.09	97–99	3	0.63
Pt 2-0M	90–98	4	28.19	92–97	4	3.93
Pt 2-3M	97–99	3	3.14	97–100	2	0.63
Pt 2-12M	92–98	3	15.45	90–99	3	2.72
Pt 3-0M	90–98	4	38.77	83–100	3	4.33
Pt 3-3M	96–99	4	6.40	96–99	3	1.61
Pt 3-12M	98–99	3	2.14	97–99	3	0.85
Pt 4-0M[b]	98–99	3	2.09	96–99	3	1.16
Pt 4-3M	99	3	3.23	98–100	3	0.89
Pt 4-12M	99	2	0.22	99	2	0.31

HVR, hypervariable region; HCV, hepatitis C virus; BMT, bone marrow transplantation; Pt, patient.
[a]Eight clones were randomly selected and sequenced for each serum sample.
[b]Patient 4 did not undergo BMT and the sera were withdrawn about the corresponding time.

TABLE II. Nucleotide Substitutions (δ) and Its Rate of HVR of HCV Between Pre-BMT and 3 Months after BMT, and Between 3 and 12 Months after BMT

Patient	δ (site^{-1})	δ rate (site^{-1} year^{-1})
pre-BMT to 3 months after BMT		
Patient 1	0.177	0.708
Patient 2	0.156	0.624
Patient 3	0.168	0.672
Patient 4[a]	0.041	0.164
3 to 12 months after BMT		
Patient 1	0.095	0.127
Patient 2	0.028	0.038
Patient 3	0.054	0.072
Patient 4	0.055	0.073

HVR, hypervariable region; HCV, hepatitis C virus; BMT, bone marrow transplantation.
[a]Patient 4 did not undergo BMT.

DISCUSSION

The quasispecies nature of HCV, like most RNA virus, is well recognized [Martell et al., 1992]. The complexity of HCV quasispecies may vary with certain conditions, such as the immune status of the hosts, the nature of virus itself, the severity of liver diseases [Honda et al., 1994], and the intervention of interferon therapy [Kanazawa et al., 1994]. The basic mechanism of its evolution may be the additive effects of immune selective pressure of the host [Kato et al., 1993], and the continuous accumulation of viral mutations [Kurosaki et al., 1994].

In this study, the nucleotide diversity of HVR of HCV in the pre-BMT sera of the three BMT patients was much higher than that of the other sera of the same individuals. At the pre-BMT time point, the patients' white counts and the cellular immune response started to decline when conditioning chemotherapy was administered 1 week before BMT. The high nucleotide diversity of HCV HVR may reflect the replication of various strains of HCV. Perhaps the administration of conditioning therapy resulted in the loss of host immune pressure, and allowed many minor strains in the liver to emerge. A minor strain(s) might then dominate and HCV quasispecies became relatively homogeneous in the sera withdrawn at 3 months after BMT. When the nucleotide diversity of the sera drawn 3 months and 12 months after BMT were compared, the nucleotide diversity of HCV quasispecies of the three BMT patients was found to range approximating to Patient 4's at 12 months after BMT. Patient 4's HCV quasispecies nucleotide diversity ($0.22–3.23 \times 10^{-2}$/site) may provide a reference for HCV-infected cases without immunosuppression. A previous study of HCV-infected children showed their nucleotide diversity ranged from 0.11×10^{-2} to 7.95×10^{-2}/site during a 3-year interval [Ni et al., 1997]. These data also provided the background references for nucleotide diversity in cases without immunosuppression.

This finding adds to data from previous observations in liver transplantation patients, from whom heavily viral-loaded livers were removed and minor amounts of extrahepatic HCV replicates back to the liver. Thus, a relatively homogeneous viral population was observed. For the BMT patients, the liver was not touched and the huge amount of viruses in the liver had the chance to flourish. This might be the reason we saw an initial stage of high nucleotide diversity of HCV quasispecies after immunosuppression. However, after a certain period, a dominant strain still emerges, as in the case in liver transplantation, thus made a homogeneous quasispecies.

The same observation applied to the nucleotide substitution rate, which was very high between pre-BMT and 3 months after BMT (0.624–0.708/site per year). The nucleotide substitution rate was relatively low between 3 and 12 months after BMT ($4–10 \times 10^{-2}$/site per year). Previous reports showed considerable differences in the nucleotide substitution rate of the HVR among cases of chronic HCV infection with acute exacerbation (0.14–0.91/site per year), whereas in the quiescent stage the rate was 0.013–0.121/site per year [Kurosaki et al., 1993; Sakamoto et al., 1994]. The data differed from our data in the BMT stage and the post-

BMT stage. In general, the evolutionary rate of HCV whole genome in infected humans was estimated to be 1.92×10^{-3}/site per year [Ogata et al., 1991; Mizokami et al., 1994]. Because our target in this study was the HVR region, which is the part of HCV genome most susceptible to change, the nucleotide substitution rate was thus comparably high.

The importance of host immune status in the evolution and diversity of HVR of HCV quasispecies was demonstrated in this study. This observation conforms to the previous report on HCV quasispecies changes in an agammaglobulinemic patient [Kumar et al., 1994]. However, in agammaglobulinemic patients, the main deficiency is humoral immunity whereas in BMT patients, cellular immunity is seriously suppressed. In this study, anti-HCV OD value were high (>2.0) after BMT. Before BMT, the anti-HCV OD varied. Humoral response to HCV antigen seemed to be reserved with immunosuppression for BMT. Our previous study about the evolution of HVR in mother-to-infant transmission of HCV [Ni et al., 1997] showed HCV quasispecies during infancy, perhaps with an immature immune status, were relatively homogeneous; however, the nucleotide diversity increased as time of infection increased. Lawal et al. [1997] demonstrated HCV-infected recipients of liver transplantation had a homogeneous quasispecies, fewer nucleotide changes, fewer amino acid changes, and lower replacement to silent mutations than the untreated HCV-infected patients. All these data implied that if the immunity of the hosts is deficient, one dominant HCV strain will emerge and the viral populations will become relatively homogeneous. Whenever the host immune status recovers, the quasispecies becomes relatively diverse. Thus the virus may escape the host's immune surveillance. The interesting finding in the study of these BMT patients was the initial high nucleotide diversity stage when immunosuppressive agents were just administered.

HCV RNA viral titer was highest when white cell count reached its nadir. This finding reflected the host immune status affected by the replication of HCV. This observation is consistent with those made in hemophiliac patients of HIV and HCV coinfection [Eyster et al., 1994], liver transplantation patients [Chazouilleres et al., 1994], and in pediatric renal transplant patients [Kudo et al., 1995]. All of the above patients had an elevated HCV RNA titer due to a compromised immune status.

In conclusion, the nucleotide diversity and substitution rate of HCV quasispecies depend much on the host's immune status. A drastic change in immune system, such as the immunosuppression during BMT, will speed the nucleotide substitution but select one dominant strain. Once the immune system reconstitutes, the diversity and substitution will resume its pace of evolution.

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